LIPID-DRUG FORMULATIONS AND METHODS FOR TARGETED DELIVERY OF LIPID-DRUG COMPLEXES TO LYMPHOID TISSUES

CROSS REFERENCE

This application claims the benefit of Provisional Patent Application No. 60/440,220, filed January 14, 2003.

TECHNICAL FIELD

The present invention relates to the compositions and related methods for the delivery of pharmaceutical agents to the lymphoid system, and in particular, to the lymphoid-specific delivery of various lipid-pharmaceutical and lipid-biological complexes.

BACKGROUND

Since the emergence of the Human Immunodeficiency Virus/ Autoimmune Deficiency Syndrome ("HIV/AIDS") epidemic in the 1980's, the total number of HIV/AIDS-related death is estimated to be 17.5 million globally. More recent global statistics of the HIV/AIDS epidemic suggest a grim picture. For the year 2003, approximately 5 million people are estimated to have been newly infected with the retrovirus, HIV, involving 4.2 million adults and 700,000 children under fifteen years of age. The number of people who are living with the HIV/AIDS in 2003 is estimated to be 40 million, of which 37 million are adults and 2.5 million are children. For the year 2003, approximately 3 million HIV/AIDS-related deaths are estimated, which includes 2.5 million adults and 500,000 children. Although the estimates for 2003 are lower than those published for 2002, the number of people living with HIV/AIDS is not decreasing, nor is there a decline in the epidemic. From1998 to 2002, the estimated number of deaths among persons with AIDS declined 14%. However, AIDS prevalence, or the number of persons living with AIDS, continues to increase. At the end of 2002, an estimated 384, 906 persons in the United States were reported to be living with AIDS. The term HIV/AIDS may refer to three categories of cases: (1) new diagnosis of HIV infection only; (2) new diagnoses of HIV infection with later diagnoses of AIDS; and (3) concurrent diagnoses of HIV infection and AIDS.

In order to treat HIV/AIDS-afflicted patients, an aggressive form of therapy was implemented in 1996, known as the "highly active anti-retroviral therapy" or

("HAART"), in which a plethora of drugs are administered to patients systemically. The clinical urgency for drugs having more potent anti-HIV effects has motivated the development of various types of anti-HIV drugs, including nucleoside analogs (e.g., dideoxynucleoside derivatives, including 3'-azido-3'-deoxythymidine ("AZT"), dideoxy cytidine ("ddC"), and dideoxy inosine ("ddI"), protease inhibitors, and phosphonoacids (e.g., phosphonoformic and phosphonoacetic acids). Many of these anti-HIV agents are lipid-derivatized or incorporated into liposomes prior to systemic administration (Hostetler, KY et al., *Methods of treating viral infections using antiviral liponucleotides*, Ser. No 09/846,398, US 2001/0033862; U.S. Patent No. 5,223,263; Hostetler, KY et al., *Lipid derivatives of phosphonoacids for liposomal incorporation and method of use,* U.S. Patent No. 5,194,654; Gagne J. F. et al., *Targeted delivery of indinavir to HIV-1 primary reservoirs with immunoliposomes*, Biochem Biophys Acta. 1558(2):198-210, [Feb. 2002]). For example, some anti-HIV drugs were encapsulated into the aqueous core of multilamellar and polyethyleneglycerol derivatized liposomes ("PEGylated") (Bergeron, MG. et al., *Targeting of infectious agents bearing host cell proteins,* WO 00/66173 A3; Bergeron, MG. et al., *Liposomes encapsulating antiviral drugs*, U.S. Patent No. 5,773,027; Bergeron, MG. et al., *Liposome formulations for treatment of viral diseases,* WO 96/10399 A1; Gagne JF et al., *Targeted delivery of indinavir to HIV-1 primary reservoirs with immunoliposomes,* Biochem Biophys Acta. 1558(2):198-210, Feb. 2002; Dufresne I *et al., Targeting lymph nodes with liposomes bearing anti-HLA-DR Fab' fragments,* Biochem Biophys Acta. 1421(2):284-94 [1999]; Bestman-Smith J et al., *Sterically stabilized liposomes bearing anti-HLA-DR antibodies for targeting the primary cellular reservoirs of HIV-1* Biochem Biophys. Acta. 1468(1-2):161-74 [2000]; Bestman-Smith J *et al., Targeting cell-free HIV and virally-infected cells with anti-HLA-DR immunoliposomes containing amphotericin B*, AIDS 10;14(16):2457-65 [2000]; Harvie P, Desormeaux A *et al., Lymphoid tissues targeting of liposome-encapsulated 2',3'-dideoxyinosine,* AIDS 9(7):701-7 [1995]).

Development of Lipid-Drug Complexes as a Mechanism for Drug Delivery

In general, lipid-drug complexes are formed from the aggregation of lipid molecules and pharmaceutical agents, in which the lipid component is a major constituent. Lipid-drug complexes are colloidal particles that can adopt certain configurations, such as an enclosed lipid bilayer or a lipid-drug sheet-disk complex. Lipid-drug complexes, including various forms of liposomes or lipid vesicles, can be prepared by employing lipid molecules

derived from either natural sources or formed by chemical synthesis. Although lipid constituents can vary, many formulations employ synthetic products consisting of natural phospholipids, mainly phosphatidylcholine. Most of the liposome formulations approved for human use contain a phosphatidylcholine component comprising a neutral head group and fatty acyl chains of variable lengths and degrees of saturation. A fraction of cholesterol (~30 mol %) can be included in the lipid formulation in order to modulate the rigidity and to reduce the serum-induced instability caused by the binding of serum proteins to the liposome membrane.

The composition of the lipid head group and the pH of the operative environment determine whether the liposomes formed bear a negative, neutral, or positive charge on the liposome surfaces. The nature and the density of charge on the surface of liposomes influence the stability, the kinetics, and the extent of biodistribution, as well as the interaction with and uptake of liposomes by target cells. Liposomes with a neutral surface charge have a lower tendency to be cleared by cells of the reticuloendothelial system ("RES") after systemic administration and have the highest tendency to aggregate. Although negatively charged liposomes are less likely to aggregate and are more stable in suspensions relative to neutral liposomes, negatively charged liposomes are prone to nonspecific-cellular uptake *in vivo*. Negatively charged liposomes containing phosphatidylserine ("PS") or phosphatidylglycerol ("PG") were observed to be endocytosed at a faster rate and to a greater extent than neutral liposomes (Allen TM, *et al.*, *Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo*, Biochim Biophys Acta 1066:29-36 [1991]; Lee RJ, *et al.*, *Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro*, Biochem Biophys Acta 1233:134-144 [1995]). Presumably, the negative surface charge is recognized by receptors found on a variety of cells, including macrophages (Allen TM *et al.* [1991]; Lee RJ, *et al.*, *Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis*, J Biol Chem 269:3198-3204 [1994]).

The inclusion of some glycolipids, such as the ganglioside GM_1 or phosphotidylinositol ("PI"), inhibits the liposome uptake by macrophages and RES cells, and prolongs the duration of liposome circulation. A small amount of negatively charged lipids can stabilize neutral liposomes against an aggregation-dependent uptake mechanism (Drummond DC, *et al.*, *Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors*, Pharmacol Rev 51:691-743 [1999]). Positively charged, cationic liposomes,

often used as a DNA condensation reagent for intracellular DNA delivery in gene therapy, interact with serum proteins. The aggregates of liposome and serum proteins are recognized by RES cells, and the uptake by RES cells promotes clearance in the lung, liver, and spleen. This mechanism of RES-mediated clearance partly explains the low levels of *in vivo* transfection efficiency. Other factors such as DNA instability, immune-mediated clearance, inflammatory response, and non-accessibility to target tissue can also contribute to low transfection efficiency levels in mammals. High doses of positively charged liposomes can produce varying degrees of tissue inflammation (Scheule RK, *et al.*, *Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung*, Hum. Gene. Ther 8:689-707 [1997]).

Although the technology for forming primitive forms of hydrated lipid bilayer films as spherical vesicles or liposomes was developed in the 1960's, the potential for applying lipid-drug complexes as a drug-delivery system was not realized until 30 years later. In general, the lipid component of a lipid-drug complex can be modified to exhibit biodegradable or biocompatible properties so that various lipid-drug complexes can be produced to function as an ideal drug carrier. Typically, a lipid-drug complex comprises a lipid bilayer shaped in a spherical vesicle form, in which the lipid bilayer envelops a drug within the internal space of the vesicle. For example, the particular chemistry and geometry of liposomes enable an efficient delivery system that can simultaneously reduce the toxicity of therapeutics as well as enhancing the potency of the drug. The therapeutic index of the drug can be modulated in order to reduce the toxicity and/or increase the efficacy of the parent drug (Bangham AD, *Liposomes: the Babraham connection*, Chem. Phys. Lipids 64:275-285 [1993]). Similar liposome-based therapeutics have been approved for human use by the U. S. Food and Drug Administration ("FDA"). Thus, liposomes have been used as drug carriers in pharmaceutical applications since the mid-1990s (Lian, T. and Ho, R.J.Y., *Trends and Developments in Liposome Drug Delivery Systems*, J. Pharm. Sci. 90(6):667-80 [2001]).

Liposomes can be designed to have more stability both *in vitro* and *in vivo*, with improved biodistribution, and with optimized resident time of liposomes in the systemic circulation. By utilizing hydrophilic polymers to enhance the degree of surface hydration or by using steric modification strategies, the surface of a liposome membrane can be modified in order to reduce the degree of aggregation and to avoid recognition by RES cells. For example, surface modification is often done by incorporating gangliosides, such as GM_1, or

lipids that are chemically conjugated to hygroscopic or hydrophilic polymers, usually polyethyleneglycol ("PEG"). Similar to the process of protein PEGylation, in which PEG molecules are conjugated to therapeutic proteins such as adenosine deaminase, a common alderase used for the treatment of AIDS, PEG can be conjugated to the terminal amine of phosphatidylethanolamine constituting a liposome (Beauchamp C, *et al.*, *Properties of a novel PEG derivative of calf adenosine deaminase*, Adv Exp Med Biol 165:47-52 [1984]). The presence of hydrophilic polymers on the liposome membrane surface provides an additional surface hydration layer (Torchilin VP, *Immunoliposomes and PEGylated immunoliposomes: possible use of targeted delivery of imaging agents*, Immunomethods 4:244-258 [1994]). One advantage of hydrated liposomes is that such liposomes evade recognition by macrophages and RES cells as foreign particles, and therefore, precludes phagocytic clearance by these cells.

Liposome size may affect vesicle distribution and clearance after systemic administration so that increasing the vesicle size can enhance RES-mediated uptake of liposomes (Hwang K, *Liposome pharmacokinetics*, In: Ostro MJ, editor, *Liposomes: from biophysics to therapeutics*, New York: Marcel Dekker, pp. 109-156 [1987]). Whereas RES-mediated uptake *in vivo* can be saturated at high doses of liposomes or by pre-dosing with large quantities of control liposomes deficient in drug content, this strategy may not be practical for human therapeutic use because sustained impairment of the RES physiological functions may introduce adverse effects in patients (Senior J, *et al.*, *Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection*, Biochem Biophys Acta 839:1-8 [1985]). Most recent investigations have employed unilamellar vesicles, 50-100 nm in size, for systemic drug delivery applications. For example, the antifungal liposome product "AmBisome" can be formulated so that the size specification is 45-80 nm in order to reduce the RES-mediated uptake of antifungal liposomes.

Serum protein binding is an important factor that affects liposome size and increases the rate of liposome clearance *in vivo,* when administered by intravenous (IV) route. In particular, processes such as complement activation by liposomes and opsonization depend on liposome size (Devine DV, *et al.*, *Liposome-complement interactions in rat serum: Implications for liposome survival studies*, Biochim Biophys Acta 1191:43-51 [1994]; Liu D, *et al.*, *Recognition and clearance of liposomes containing phosphatidylserine are mediated by serum opsonin*, Biochem Biophys Acta 1235:140-146 [1995]). Although PEG can be incorporated into liposome formulations to reduce serum protein binding to liposomes, the

upper size limit to ensure prolonged circulation of PEG-PE liposomes is ~200 nm. Due to biological constraints, the development of relatively large (>500 nm) liposomal structures having prolonged circulating properties, by using steric stabilization methods, has not been successful. For optimizing liposome-drug delivery systems, liposome composition and size are critical considerations in that the mechanisms of biodistribution and disposition *in vivo* can vary depending on the lipid composition, the liposome size, the liposome charge, and the degree of liposome surface hydration or steric hindrance.

The route of administration may affect the *in vivo* disposition of liposomes mainly because immediately after intravenous administration, liposomes are usually coated with serum proteins, and are taken up or eliminated by circulating RES cells (Chonn A, *et al., Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes*, J Biol Chem 267:18759-18765 [1992]; Rao M, *et al., Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells*, Adv Drug Deliv Rev 41:171-188 [2000]). Plasma proteins that can interact with liposomes include albumin, lipoproteins or any high-density lipoprotein ("HDL"), low-density lipoprotein ("LDL") and cell-associated proteins. Some of these proteins such as HDL can remove phospholipids from the liposome bilayer, thereby destabilizing the liposomes. This process may potentially lead to a premature leakage or dissociation of drugs from liposomes.

As a drug delivery system, liposomes are especially promising because they can modulate the pharmacokinetics of liposome-associated and encapsulated drugs, which is not possible with non-lipid-associated or "free" drugs (Allen, T.M *et al.* [1991]; Hwang, K. [1987]; Allen T, *et al., Pharmacokinetics of long-circulating liposomes*, Adv Drug Del Rev 16:267-284 [1995]; Gabizon A, *Liposome circulation time and tumor targeting: Implications for cancer chemotherapy*, Adv Drug Del Rev 16:285-294 [1995]; Bethune C, *et al., Lipid association increases the potency against primary medulloblastoma cells and systemic exposure of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in rats*, Pharm Res 16:896-903 [1999]). However, therapeutic applications of systemically (IV) administered liposomes have been limited by the rapid clearance of liposomes from the bloodstream and uptake by RES cells (Alving C, *et al., Complement-dependent phagocytosis of liposomes: Suppression by 'stealth' lipids*, J Liposome Res 2:383-395 [1992]). Incorporation efficiencies for loading many pH-titratable drugs within the interior aqueous compartment of liposomes, including some protease inhibitors such as indinavir, typically have been relatively low (Gagne, JF *et al., Targeted delivery of indinavir to HIV-1 primary reservoirs*

with immunoliposomes, Biochim Biophys Acta 1558(2):198-210 [2002]). Although significant advances have been made in the field of lipid-drug formulation technology in recent years, a need for developing compositions and methods that can provide an effective pharmaceutical-delivery system, which can incorporate drugs and biomolecules or "biologicals" at high efficiency, and deliver stable lipid-pharmaceutical and lipid-biological complexes to a lymphoid tissue is recognized.

SUMMARY OF THE INVENTION

Various embodiments of the present invention are directed to lipid-pharmaceutical compositions and related methods for producing a lipid-drug complex under conditions near the neutral pH range. Optimal pH range is provided for the efficient incorporation of various lipid-drug complexes. A lipid-drug complex, such as a liposome, readily encapsulates drugs having low aqueous solubility within a neutral pH range. In some embodiments, the lipid-drug complex comprises a lipid bilayer and a lipid-soluble drug having a range of molar ratio values of lipid-to-drug from about 3:1 to about 100:1 or higher for relatively toxic drugs. Lipid-drug complexes can also be formed within a range of molar ratios from about 3:1 to about 10:1, and a range of molar ratios from about 5:1 to about 7:1. In various embodiments, biomolecules, such as nucleic acids and proteins, that can have pharmacological activities may also be incorporated within lipid vesicles. The methods for targeting lymphoid tissue involve subcutaneous administration of lipid-drug complexes and lipid-biomolecule complexes, and not by systemic administration.

Some embodiments are directed to lipid-drug complex formulations and methods for efficiently incorporating anti-HIV drugs and anti-HIV biologicals into a lipid-drug delivery vehicle. For the present methods, any number or combinations of lipid-anti-HIV drug or lipid-anti-HIV biological complexes can be subcutaneously injected into a HIV-infected mammalian subject, so that high concentrations of stable lipid-drug complexes can be preferentially delivered to the lymphoid tissue via lymphatic vessels, instead of delivering intravenously via the blood stream at lower concentrations. Relative to the non-lipid-associated, or "free," drug, the HIV-infected cells exposed to anti-HIV agents delivered by lipid-drug complexes can significantly improve the uptake of lipid-associated drugs so that lower effective drug concentrations may be administered than by conventional methods of systemic administration. Embodiments of the present invention provide a means for targeting HIV reservoirs within infected lymphoid cells located within the lymph nodes by

administering less concentrated doses of anti-HIV drugs, less frequently than currently practiced in the art, or by a combination of less concentrated doses of anti-HIV drugs and less frequent delivery. This lymphoid-targeted delivery of anti-HIV drugs provides a mode of treatment for HIV infection and AIDS. Other embodiments are directed to the lymphoid-specific delivery of anticancer drugs and anticancer biologicals to treat any lymphoid-related cancers, such as breast cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A illustrates the pH-dependent incorporation of indinavir within lipid-associated complexes, as discussed in Example 2.

Figure 1B illustrates the pH-dependent release of indinavir from lipid-associated complexes *in vivo*, as discussed in Example 2.

Figure 2A illustrates a typical time course for a virus load, and the $CD4^+$ T cell profile of macaques infected with HIV-2_{287}, as discussed in Example 3.

Figure 2B illustrates the analysis of plasma for a viral RNA profile of 27 macaques that were infected with 50 TC_{ID50} HIV-2_{287}, as discussed in Example 4.

Figure 3 illustrates the concentration-dependent inhibition of HIV-$_{287}$ replication by free (not lipid-associated) and lipid-associated indinavir, as described in Example 5.

Figure 4 illustrates a time course for plasma concentration of indinavir following the subcutaneous administration of lipid-associated and non-lipid-associated indinavir within macaques, as described in Examples 6 and 7.

Figure 5 illustrates the changes in plasma virus load and the $CD4^+$ T-cell profile monitored in two HIV-2_{287}-infected macaque at 25 weeks post-infection, as described in Example 8.

Figure 6 shows the concentration-dependent inhibition of HIV-1_{LAV} replication by the free and lipid-associated indinavir, as described in Examples 5 and 9.

Figure 7 shows *in situ* hybridization analysis of lymph node sections in indinavir treated animals with a [^{35}S]-labeled HIV-2_{287}-specific probe, as described in Example 10.

DETAILED DESCRIPTION

The embodiments of the present invention include various lipid-drug complexes, methods for producing a lipid-drug complex, and methods for employing lipid-drug complexes produced by the present invention in order to treat various clinical conditions that may affect lymphoid tissues. A "complex" can mean any mixture or aggregation that results from the formation of any type of chemical binding/bonding reaction among the constituents or components of the complex. The components of a complex may be bonded together by covalent bonds and non-covalent interactions, including ionic interactions, hydrogen bonds, Van der Waal's interactions, hydrophobic interactions, or any combination of these bonds, forces, and interactions. A "lipid-drug complex" can mean a complex in which at least one component is any form of a lipid molecule, and at least one component is any form of a pharmaceutical agent, such as an anti-viral drug, an anti-fungal drug, or an anti-cancer drug. The formation of lipid-drug complexes may also be described as a lipid-association or a lipid-incorporation. The reverse process of dissociating bonds between components of a lipid-drug complex may be described as a dissociation or a release.

The lipid-drug complexes of the present invention may adopt various types of configurations, including the spherical shape of liposomes, and various lipid-drug-sheet-disk complexes. A liposome forms generally as a vesicle comprising a lipid bilayer membrane with an aqueous internal space. A lipid-drug complex can be in a non-vesicular bilayered configuration, or can be configured as a micelle. In one embodiment, the lipid-drug complex is a unilamellar liposome. Unilamellar liposomes provide the highest exposure of drug to the exterior of the liposome, where it may interact with the surfaces of target cells. However, multilamellar liposomes can also be made. The size of a liposome-drug complex is preferably, but not necessarily, about 30 to about 150 nanometers in diameter, and more preferably about 50 to about 80 nanometers in diameter (see Table 1B, Example 2).

In one embodiment, the drug component of the lipid-drug complex can be a molecule having an anti-viral effect, such as a non-nucleoside anti-HIV drug. Other examples of anti-HIV drugs include the HIV reverse protease inhibitors: indinavir (aka Crixivan®, Merck & Co., Inc., Rahway, NJ); saquinavir (N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]-amino]butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide; MW = 670.86; aka Fortovase®, Roche Laboratories, Inc., Nutley, NJ); or nelfinavir (i.e., nelfinavir mesylate, aka Viracept®; [3S-[2(2S*, 3S*), 3a,4ab,8ab]]-N-(1,1-dimethylethyl)decahydro-2-[2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-(phenylthio)butyl]-3-isoquinolinecarboxamide monomethanesulfonate (salt), MW = 663.90 [567.79 as the free base]; Pfizer formally, Agouron Pharmaceuticals, Inc., La Jolla, CA). Other examples of antiviral drug include reverse transcriptase inhibitors, such as tenofovir disoproxil fumarate (9-[(R)-2-[[bis[[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1); MW = 635.52; aka Viread®, Gilead Sciences, Foster City, CA).

In other embodiments, the drug can be an anticancer drug, an antifungal drug, or an antibacterial drug. In other embodiments, the drug can be an immunomodulatory drug (i.e., an immunoactivator, an immunosuppressant, or an antiinflammatory), such as cyclosporin, steroids and steroid derivatives. Various embodiments of the present invention include the lipid incorporation or lipid-association of a number different types of drugs, and combinations of drugs. For example, liposomes can incorporate a large number of one or more different anti-HIV drugs, anti-fungal drugs, antibacterial drugs, and anti-cancer drugs. In addition to the incorporation of pharmaceutical agents, such as drugs, various types of biologicals may also be included within the interior space of lipid vesicles such as liposomes. The term biologicals include a large number of different biomolecules, such as single or double-stranded forms of DNA and RNA, proteins, glycoproteins, and other biopolymers that can be incorporated by the various method embodiments of the present invention these embodiments are described, throughout this disclosure, using the drug indinavir as an example. Examples of biologicals that can be lipid-incorporated include anti-sense RNAs, single-stranded inhibitory RNA (siRNA), proteins, ribozymes, nucleic acid polymers, proteases, and antibodies. Other embodiments are directed to various drugs and biologicals that may be delivered to lymphoid tissues by lipid vesicles for the treatment of HIV infection and AIDS. Other embodiments are directed to various types of drugs and various types of biologics that may be delivered to lymphoid tissues for the treatment of metastatic breast

cancer. For example, drugs (e.g., taxol and DNA intercalating agents) and biologics (e.g., anti-her-2/neu antibodies and anti-sense RNAs) having anti-cancer or anti-proliferative effects may be incorporated within lipid vesicles as lipid-drug and lipid-biological complexes.

The present methods provide a lipid-based, drug delivery vehicle for drugs and biologicals that exhibit an increase in membrane affinity within a neutral or physiological pH range. The phrase "a drug having low aqueous solubility within a neutral pH range" means a drug that is more lipophilic within a neutral pH range spanning a range near pH 5.5 to about pH 8.0, and more preferably within a range from pH 7.0 to about pH 7.4 (see Figure 1A and 1B). Drugs such as indinavir, nelfinavir, saquinavir, viread (described above and see Table 1B, Example 2) are drug that have low aqueous solubility within a neutral pH range, and therefore are included in the invention. For example, nelfinavir mesylate is a white to off-white amorphous powder, which is slightly soluble in water at pH <4, and it is freely soluble in methanol, ethanol, isopropanol and propylene glycol.

The present invention does not depend on a particular chemical or biochemical mechanisms by which the inventive lipid-drug formulations are obtained, or by which the drug is released to target lymphoid cells. However, it is thought that the complementary structure of the drug, e.g., indinavir (as it assumes lipophilic form at pH 7.4) intercalates within the lipid bilayer. For example, indinavir has an aniline group which is an aromatic six-membered ring that includes 5 carbons and 1 nitrogen. Amines usually have a high pK, which is indicative of a base. However, aniline has a relatively low dissociation constant (pK = 4.76), indicative of a weak acid. The protonation of the weak base (the amine group) results in increased aqueous solubility of the drug, which can promote the packaging of the drug at acidic pH range. Titration back to a neutral pH range decreases the aqueous solubility of the drug and increases its lipophilicity, and results in the association between the drug and the lipid bilayer of the liposome. It is thought that endocytosis of the liposomes by cells results in the sequestration within the intracellular acidic vesicles, and that the acidic pH of the vesicles increases the aqueous solubility of the drug, resulting in its release from the liposome into the cell. The mechanism for the drug release from the endocytic vesicle is unknown.

By way of example, indinavir is an HIV protease inhibitor, typically formulated as a sulfate salt of N-(2(R)-hydroxy-1(S)-indanyl)-2(R)-phenylmethyl-4-(S)-hydroxy-5-(1-(4-(3-pyridyl-methyl)-2(S)-N'-(t-butylcarboxamido)-piperazinyl))-

pentaneamide ethanolate. (e.g., U.S. Pat. No. 5,413,999). Indinavir in pill form (Crixivan®, Merck & Co., Inc., Rahway, NJ) is typically administered to AIDS patients at a dosage of 800 mg, three times a day. In contrast to the embodiments of the present invention, the U.S. Pat. No. 5,413,999 discloses that indinavir can be taken in a pill form (not lipid-associated), and that the drug should be delivered systemically and not preferentially to lymphoid tissues. Indinavir has about 1000-fold lower solubility in water at neutral pH 7 than at acidic pH 3-4. However, by formulating the lipid-indinavir complex, at a lipid-to-drug molar ratio range from about 5:1 to about 10:1, within a neutral pH range where the aqueous solubility of indinavir is relatively low, 80-100% of an indinavir preparation is incorporated into the liposomes, compared to much lower efficiencies obtained at pH 3-4 (less than 30%), or by other known methods (e.g., Gagne JF *et al.*, *Targeted delivery of indinavir to HIV-1 primary reservoirs with immunoliposomes*, Biochem Biophys Acta 2002 Feb 1;1558(2):198-210).

The inventive method involves dissolving the drug in a solvent. In some embodiments, the drug can be dissolved in an aqueous solvent, such as water or a biocompatible buffer solution, including phosphate-buffered saline, HEPES, TRIS, or the like. A detergent, such as Tween 80, can also be employed in conjunction with an aqueous solvent. Dissolving the drug can be accomplished in the presence, or in the absence or before the addition of the lipids. In other embodiments, the drug is more effectively dissolved in an organic solvent, such as dimethyl sulfoxide (DMSO), methanol, ethanol, propanol, propane glycol, butanol, isopropanol, pentanol, pentane, a fluorocarbon (e.g., freon), or an ether. However, the use of carcinogenic organic solvents, such as toluene, benzene, or methylene chloride, should be avoided.

Examples of useful lipids include any vesicle-forming lipid, such as, but not limited to, phospholipids, such as phosphatidylcholine (hereinafter referred to as "PC"), both naturally occurring and synthetically prepared, phosphatidic acid ("PA"), lysophosphatidylcholine, phosphatidylserine ("PS"), phosphatidylethanolamine ("PE"), and sphingolipids, phosphatidyglycerol ("PG"), spingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides and the like used either singularly or intermixed such as in soybean phospholipids (e.g., Asolectin, Associated Concentrates). The PC, PG, PA and PE can be derived from purified egg yolk and its hydrogenated derivatives.

In addition, other lipids such as steroids, cholesterol, aliphatic amines such as long-chained aliphatic amines and carboxylic acids, long chained sulfates and phosphates, diacetyl phosphate, butylated hydroxytoluene, tocopherols, retinols, and isoprenoid

compounds can be intermixed with the phospholipid components to confer certain desired and known properties onto the formed vesicles. In addition, synthetic phospholipids containing either altered aliphatic portions such as hydroxyl groups, branched carbon chains, cycloderivatives, aromatic derivatives, ethers, amides, polyunsaturated derivatives, halogenated derivatives or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quarternary amine, sulfate, sulfonate, carboxy, amine, sulfhydryl, or imidazole groups. Combinations of such groups can be either substituted or intermixed with the above-mentioned phospholipids. It will be appreciated from the above that the chemical composition of the lipid components prepared by the present method can be varied greatly without appreciable diminution of percentage drug capture, although the size of a vesicle can be affected by the lipid composition. Saturated synthetic PC and PG, such as dipalmitoyl can also be used. Other amphipathic lipids that can be used, advantageously with PC, are gangliosides, globosides, fatty acids, stearylamine, long chain alcohols, and the like. PEGylated lipids, monoglycerides, diglycerides, triglycerides can also be included. Acylated and diacylated phospholipids are also useful.

By way of further example, in some embodiments, useful phospholipids include egg phosphatidylcholine ("EPC"), dilauryloylphosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DOPC"), dipalmitoylphosphatidylcholine ("DPPC"), distearoylphosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoylphosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoyl phosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoyl phosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl phosphatidylcholine ("SPPC"), dioleoylphosphatidylycholine ("DOPC"), dilauryloylphosphatidylglycerol ("DLPG"), dimyristoylphosphatidylglycerol ("DMPG"), dipalmitoylphosphatidylglycerol ("DPPG"), distearoylphosphatidylglycerol ("DSPG"), distearoyl sphingomyelin ("DSSP"), distearoylphophatidylethanolamine (DSPE), dioleoylphosphatidylglycerol ("DOPG"), dimyristoyl phosphatidic acid ("DMPA"), dipalmitoyl phosphatidic acid ("DPPA"), dimyristoyl phosphatidylethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), dimyristoyl phosphatidylserine ("DMPS"), dipalmitoyl phosphatidylserine ("DPPS"), brain phosphatidylserine ("BPS"), brain sphingomyelin ("BSP"), and dipalmitoyl sphingomyelin ("DPSP").

In one embodiment, phosphatidylcholine and cholesterol at 3:1 molar ratio are employed. However, any suitable molar ratio of a non-steroidal, lipid-steroidal lipid (e.g.,

cholesterol) mixture can optionally be employed that promotes the stability of a particular lipid-drug complex during storage and/or delivery to a mammalian subject.

Mixing the drug and lipids can be by any useful known technique, for example, by sonication, vortexing, extrusion, microfluidization, homogenization, and use of a detergent, which may be later removed, e.g., by dialysis. The drug and lipid are mixed at a lipid-to-drug molar ratio of about 3:1 to about 100:1 or higher (especially useful for relatively more toxic drugs), and more preferably about 3:1 to about 10:1, and most preferably about 5:1 to about 7:1. If an organic solvent is used in the inventive method for producing a lipid-drug complex, such as a liposome, the organic solvent can be removed, after the mixing of the drug and lipids, by any suitable means of removal, such as evaporating by vacuum, or by the application of heat, for example by using a hair dryer or an oven, or hot ethanol injection (e.g., Deamer, United States Patent No. 4,515,736), as long as the lipid and drug components are stable at the temperature used. Dialysis and/or chromatography, including affinity chromatography can also be employed to remove the organic solvent. Drug hydration is performed with water or any biocompatible aqueous buffer, e.g., phosphate-buffered saline, HEPES, or TRIS, that maintains a physiologically balanced osmolarity. Rehydration of liposomes can be accomplished, simultaneously by removing the organic solvent, or alternatively, can be delayed until a more convenient time for using the liposomes (See, e.g., Papahadjopoulos *et al.*, United States Patent No. 4,235,871). The shelf life of hydratable (i.e., "dry") liposomes is typically about 8 months to about a year, which can be increased by lyophilization.

Embodiments of the present invention provides a lipid-drug complex, for example, a liposome, comprising a lipid bilayer and a drug, within a neutral pH range, the molar ratio of the lipid-to-drug in the liposome is within a range of about 3:1 to about 100:1 or higher (especially for relatively more toxic drugs), and more preferably within a range of about 3:1 to about 10:1, and most preferably about 5:1 to about 7:1. The inventive lipid-drug complexes are also characterized by the fact that the drug substantially dissociates from the liposome at a range of about pH 5.0 to about pH 5.5. The term "substantially dissociates" means that approximately 50% or more of the drug that was associated with the lipid in a lipid-drug complex, at a first pH value, dissociates from the lipid-drug complex at a second pH value, e.g., at about pH 5.0 to about pH 5.5.

The lipid-drug complex, e.g., a liposome, is administered to a subject by any suitable means, for example by injection. Injection can be intrarterial, intravenous,

intrathecal, intraocular, subcutaneous, intramuscular, intraperitoneal, or by direct (e.g., stereotactic) injection into a tumor or other types of lesion. Subcutaneous or intramuscular injection are preferred for introducing the lipid-drug complex into lymphatic vessels. The lymphoid tissue is a lymph node, such as an inguinal, mesenteric, ileocecal, or axillary lymph node, or the spleen, thymus, or mucosal-associated lymphoid tissue (e.g., in the lung, lamina propria of the of the intestinal wall, Peyer's patches of the small intestine, or lingual, palatine and pharyngeal tonsils, or Waldeyer's neck ring). Injection is by any method that drains directly, or preferentially, into the lymphatic system as opposed to the blood stream. Most preferred mode of administration is by way of subcutaneous injection, typically employing a syringe needle gauge larger than the lipid-drug complex. Intraperitoneal injection can also be used. Typically, the injectate volume (generally about 1-5 cm^3) is injected into the subject's arm, leg, or belly, but any convenient site can be chosen for subcutaneous injection. Because the drug subcutaneously administered, the drug enters the lymphatic system prior to entering the systemic blood circulation. Some advantages include: (1) the distribution throughout the lymphoid system and localization into lymph nodes, (2) the preclusion or minimization of protein-mediated destabilization of lipid-drug complexes, and (3) the delivery of indinavir at concentrations that cannot be achieved with a soluble form of the drug (not lipid-associated or "free") administered by any route of administration.

Typically, for methods directed to treating HIV/AIDS, the frequency of injection is most preferably once per week, but more or less (e.g., monthly) frequent injections can be given as appropriate. The present invention facilitates a treatment regimen that can involve a convenient weekly injection rather than multiple drug doses daily, as practiced typically in current AIDS treatment regimes. This feature may lead to improved patient compliance with the full course of treatment for some individual patients.

The embodiments discussed can be further supported in the following examples. In Example 1, the methods employed in the various embodiments of the present invention are provided. In Example 2, the experimental data supporting the pH-dependence of lipid-drug association/ incorporation efficiency is provided. Also provided is the data supporting the pH-dependent efficiency of drug release from lipid-associated complexes. Also, in Example 2, the effect of pH on the solubility and lipophilicity of a drug (indinavir) is provided (Table 1A), and the relative sizes and degrees of lipid association for various types of drugs are provided (Table 1B). In Example 3, data supporting enhanced levels of drug delivery to lymphoid tissues are provided by comparing indinavir concentrations in human lymph

node (LNMC) and peripheral blood mononuclear cells (PBMCs). In Example 3, a typical time course following HIV-2 infection in monkeys is presented (Figure 1A). In Example 4, a time course for plasma concentration of indinavir following the subcutaneous administration of lipid-associated and non-lipid-associated indinavir within macaques is provided. In Example 5, the effect of lipid association on the ability of indinavir to inhibit HIV-2_{287} Replication is provided. In Example 6, a plasma time course profile of free versus lipid-associated indinavir in macaques is provided. In Example 7, the effect of lipid-drug complexes on enhanced accumulation of indinavir in lymph nodes is provided. In Example 8, the effect of lipid-indinavir complex on HIV-2_{287} infected macaques is provided. In Example 9, the effect of lipid association on the inhibition of HIV-1 replication in human peripheral blood mononuclear cells is provided. In Example 10, the reduction of HIV viral load in infected macaques by the accumulation of liposome-indinavir complexes in lymphoid tissues is provided.

EXAMPLES

Example 1

Methods of the Present Invention

Lipid-drug complex preparation and characterization: Routinely, for drug incorporation studies, 1 millimole of the drug (e.g., indinavir, saquinavir, nelfinavir, or tenofovir disoproxil fumarate) was dissolved in 1 mL of ethanol and mixed together with 5 mmoles of lipids (e.g., phosphatidylcholine [egg]: cholesterol [3:1, mol/mol]) dissolved in $CHCl_3$:ethanol (1:1, v/v). The mixture was rotor-evaporated under N_2 and reduced pressure. Then it was resuspended in sterile phosphate-buffered saline (PBS, pH 7.4), to a final lipid concentration of 50 mM, and homogenized with a probe sonicator until a uniform particle size of about 50 nm to about 80 nm diameter was reached. Sonication is preferably done until sonicated unilamellar vesicles that are stable at their minimum diameter are obtained. Typically, it took less than 30 min of sonication under aseptic conditions to achieve a 50–60 nm lipid-drug complex size in a relatively narrow distribution. The size and zeta potential (surface potential at hydrodynamic plane) of the lipid-drug complex was monitored using a Malvern Zetasizer 5000 operating at photon correlation spectroscopy mode and electrophoretic mode, respectively. In the case where a negative membrane charge is needed, about 20 mole % of phosphatidylglycerol (exhibits a net negative charge at neutral pH) can be added to the lipid mixture, solubilized in $CHCl_3$:ethanol (1:1, v/v). The rest of the preparation procedure remains the same.

The sterility and endotoxin contamination of the preparation, an important safety consideration, was routinely monitored as part of strict cGMP/cGLP guidelines. To

ensure sterility and prevent underestimation of endotoxin that binds to lipids, the lipid-drug complex preparations were subjected to the blood agar culture test for 7 days at 37°C for detection of microbial contamination. This provided a stringent evaluation of sterility and bacterial contamination to ensure the consistent quality of the lipid-drug complex.

The degree of drug incorporation into the lipid-drug complex was determined by subjecting a small fraction of the preparation to size-exclusion chromatography using a Biogel A-0.5 M media (1x10 cm). At the flow rate of 1 ml/min of PBS, lipid-associated indinavir was well separated from free drug. By analyzing the amount of fraction in the lipid-associated complex, and free form with respect to the total amount loaded onto the column, the percentage of drug association was determined. (Lian, T. and Ho, R.J.Y., *Recent trend and progress in liposome drug delivery systems; an invited minireview*, J Pharm Sci 90:667-680 [2001]). To determine pH-dependent drug dissociation from the lipid-drug complex, the lipid-drug complex (originally prepared in pH 7.4 and free of unincorporated drug) can be incubated at, e.g., pH 7, 6, 5.5, 5, 4, 3.5 for 30 min. Subsequently, the mixture can be subjected to the degree of drug incorporation analysis described above. For animal studies, the drug-lipid complex formulations were further analyzed for sterility and endotoxin contamination. To ensure sterility and prevent underestimation of endotoxin that binds to lipids, the lipid-drug complex preparations were subjected to a blood agar culture test for 7 days at 37°C for detection of microbial contamination.

Determination of virus-infected cell frequency in peripheral blood and lymph node mononuclear cells by virus coculture assay: From the macaques, isolate PBMCs were isolated from 10-ml blood samples at twice-weekly intervals after HIV-2_{287} inoculation. About 1–2 x 10^6 PBMCs/ml of blood were routinely isolated, allowing the performance of coculture assays that minimally require about 1 x 10^6 PBMCs.

HIV-2_{287} was originally isolated from the lymph node of a macaque with the clinical manifestations of AIDS. The macaque had been inoculated with HIV-2_{EHO} which had been passaged twice in macaques. A stock was prepared by growing the primary isolate in $CD8^+$-depleted, phytohemagglutinin-stimulated macaque PBMC. (Ho RJ, Agy MB, Morton WR, et al. *Development of a chronically catheterized maternal-fetal macaque model to study in utero mother-to-fetus HIV transmission: a preliminary report*. J Med Primatol 1996;25 (3):218-24; Ho RJ, Larsen K, Kinman L, et al. *Characterization of a maternal-fetal HIV transmission model using pregnant macaques infected with HIV-2(287)*. J Med Primatol

2001;30 (3):131-40). The inoculum was prepared by diluting the stock in complete tissue culture medium.

Briefly, duplicate samples of the serially diluted PBMCs isolated from the blood in a fixed volume (2 ml) were added to 10^6 human lymphoblasts in 24-well tissue culture plates. The human lymphoblasts were generated by first depleting $CD8^+$ cells from the PBMCs and then stimulating the lymphocytes (i.e., PBMCs) for 3 days with 1 μg/ml PHA-P and 20 IU/ml IL-2 in RPMI containing 10% NHu serum. The $CD8^+$ cell-depleted macaque PBMCs were sequentially diluted (in 1:5 ratios) starting from 10^6 cells per well. The feeder cells, human lymphoblasts, remained constant at 10^6 per well. The cell mixture was incubated for 21 days with the cells being fed with fresh culture medium once every week. The presence of HIV-infected macaque PBMCs was visually screened every other day for syncytia formation and verified for infection on days 14 and 21 by assaying for the HIV-2 p27 core antigen in supernatant using a sandwich antigen ELISA. The end-point dilution at which minimally detectable HIV antigen is found can be used to estimate the frequency of virus-infected cells per 10^6 macaque PBMCs. Typically, by about day 10–13 following infection with a dose of 10 $TC_{ID_{50}}$ HIV-2_{287}, the virus-infected cells peaked at about 25,000 per 10^6 PBMCs, an extremely high frequency of virus-infected cells (~0.1-1% of total PBMCs) in the periphery. (Ho, R.J.Y. *et al.*, *Suppression of maternal virus load with AZT, DDI, and indinavir combination therapy prevents mother-to-fetus HIV transmission in macaques*, JAIDS, 25, 140-149 [2000]; Ho, R.J.Y. *et al.*, *Development of a chronically catheterized maternal-fetal macaque model to study in utero mother-to-fetus HIV transmission - a preliminary report. An invited article*, J Medical Primatol 25, 218-224 [1996]).

Analysis of viral RNA and DNA in blood, lymph nodes, and other tissues: Tissues collected (lymph nodes, thymus and spleen, brain) from macaques at the time of euthanasia were assayed for drug level, as described herein below, and analyzed by immunohistochemistry, RNA- and DNA-PCR to quantitate viral load and distribution of virus in these tissues. For DNA- and RNA-PCR analyses as well as virus coculture, fresh or flash-frozen (stored at –80°C) tissues were used. For immunocytochemistry, *in situ* hybridization, and other histological analyses were conducted; the tissues were fixed following established procedures.

Briefly, tissues were be fixed in 4% neutral buffered and deionized paraformaldehyde, were embedded in paraffin wax, sectioned (5 μm) and stained with hematoxylin and eosin for routine histological examination. In addition, lymph node tissues were preserved in

Streck Tissue Fixative (STF; Streck Laboratories, Omaha, NE), a citrate-based, non-cross-linking fixative suitable for permeating dense tissues, maintaining the integrity of nucleic acids, and conserving antigenic structure of cell-surface molecules. A fraction of the tissues were used to isolate lymph node leukocytes by forcing the tissues through an 80-μM wire mesh and layering onto histopaque 1077 (Sigma, St. Louis, MO) discontinuous density gradients to isolate PBMC or lymph node mononuclear cells (LNMC) (Brodie, S.J., *et al.*, *Pediatric AIDS-associated lymphocytic interstitial pneumonia and pulmonary arterio-occlusive disease: Role of VCAM-1/VLA-4 adhesion pathway and human herpesviruses*, Am J Pathol 154:1453-1464 [1999a]; Brodie, S.J. *et al.*, *In vivo migration and function of transferred HIV-1-specific cytotoxic T cells*, Nat Med 5(1):34-41 [1999b]; Brodie, S.J. *et al.*, *HIV-specific cytotoxic T lymphocytes traffic to lymph nodes and localize at sites of HIV replication and cell death*, J Clin Invest 105:1407-1417 [2000b]). LMNC and PBMC were fixed and permeated (to preserve intracellular nucleic acid) with Permeafix (Ortho Diagnostics, Raritan, NJ) (500 μl/10^6 cells), a non-aldehyde, non-cross-linking, water-soluble fixative.

Total RNA was isolated from plasma and lymphoid tissues using a Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN). Viral RNA was measured using a quantitative, internally-controlled RNA PCR to estimate the number of HIV-2 copies/ml of sample (Watson A, Ranchalis J, Travis B, et al., *Plasma viremia in macaques infected with simian immunodeficiency virus: plasma viral load early in infection predicts survival,* J Virol 1997;71 (1):284-90) for some of the experiments and real-time quantitative PCR. The two methods have been validated to ensure consistency in estimating viral RNA concentration in plasma and tissues. Briefly, reverse transcription and PCR were performed as described. Because semi-quantitative methods have been well-documented (Watson, et al., 1997), only details on RT-QPCR are described below.

Real-time quantitative fluorescent probe PCR (TaqMan): The TaqMan PCR system was employed in a real-time automated PCR assay for quantifying HIV-1 or HIV-2 (e.g., HIV-2_{287}) DNA and RNA in plasma, PBMC, and/or a variety of solid tissues for viral load determination in HIV-infected subjects. (Brodie *et al.*, [2000a–b]; Mostad, S.B. *et al.*, *Cervical shedding of cytomegalovirus in human immunodeficiency virus type 1-infected women*, J Med Virol 59:469-473 [1999]; Mostad, S.B. *et al.*, *Cervical shedding of herpes simplex virus in human immunodeficiency virus-infected women: effects of hormonal contraception, pregnancy, and vitamin A deficiency*, J Infect Dis 181:58-63 [2000]; Krone, M.R. *et al.*, *Herpes simplex virus type 2 shedding in human immunodeficiency virus-negative men who have sex with men: frequency, patterns, and risk factors*, Clin Infect Dis 30:261-267 [2000]; Wald, A. *et al.*, *Reactivation of*

genital herpes simplex virus type 2 infection in asymptomatic seropositive persons, N Engl J Med 342:844-50 [2000]; Zerr, D.M. *et al.*, *Sensitive method for detection and quantification of human herpesviruses 6 and 7 in saliva collected in field studies*, J Clin Microbiol. 38(5):1981-83 [2000]; Ryncarz, A.J. *et al.*, *Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples*, J Clin Microbiol 37:1941-1947 [1999]). The real-time RT-PCR assay for detecting HIV *gag* RNA has been validated.

Real-time PCR is a technology that offers advantages over conventional methods, including quantitation of product copy numbers following each PCR cycle (facilitating mathematical calculation of precise copy numbers in the original sample) and markedly reduced susceptibility to contamination. This technology is highly quantitative and reproducible, and allows the consistency to perform a large volume of sample collections.

DNA and RNA were extracted from 400 μl of plasma using acid phenol (pH 4): chloroform: isoamyl alcohol (48:24:1). The specimens were eluted into 100 μl of 10 mM Tris (pH 8.0) and 20 μl of nucleic acid was used for each PCR and RT-PCR reaction. Tissues and cells were first treated with proteinase K before extraction. One to 2 μg of total cellular DNA or RNA were used for each PCR. For RNA, the nucleic acid was reverse-transcribed and amplified in a one-step reaction (Perkin Elmer, Multiscribe) (Brodie *et al.*, 2000b). The conditions and controls for the TaqMan PCR were similar to those described in Brodie et al. (2000a–b). The design of specific primers and fluorescent probes for HIV-2 *gag* DNA and RNA were based on HIV-2_{EHO} sequences (Rey-Cuille, M.A. et al., HIV-2_{EHO} isolate has a divergent envelope gene and induces single cell killing by apoptosis, Virology 202:471-6 [1994]):

Primers EHOTAQ-F': TTATTCCCACCTGCCGCTAA (SEQ ID NO:1);
EHOTAQ-R': CTGCCCCGAACTTCTTCTCTT (SEQ ID NO:2);
and Probe EHOTAQ-P': CCCCCAACCTTAAATGCCTGGG (SEQ ID NO:3).

Liquid hybridization PCR: A semiquantitative liquid hybridization PCR assay was also used to detect HIV-2, as a 'confirmation' assay to real-time PCR methods. The assay is capable of detecting a single virus copy per sample and is similar to what has been reported for HIV-1. (Brodie *et al.* [1999] and [2000b]). Nucleic acid was extracted from proteinase K-treated tissues. For vRNA, the nucleic acid was heated, cooled, and cDNA was synthesized using random hexamer primers. Sequence-specific primers were used to amplify the cDNA and the amplified viral sequence was subsequently

detected by liquid hybridization using a [^{32}P]-labeled oligonucleotide probe specific for a conserved internal region of the amplified viral product. Electrophoresis was performed in a 6% polyacrylamide gel, and the gel was dried for autoradiography. Each autoradiograph band was compared with a dilution curve containing 5, 50, 500, and 5000 copies of viral RNA, respectively. Each cDNA and PCR reaction contained both positive and negative controls. All samples that were PCR-negative for virus were confirmed to be inhibitory or non-inhibitory by performing an additional PCR with 10^3 copies of viral cDNA. Samples that failed to support amplification of the input substrate were denoted as inhibitory. All others were reported as samples void of viral RNA. Viral copy numbers were determined using the computer program QUALITY, which is based on the number of amplifications and the number of positive results at each dilution (Rodrigo, A.G. *et al.*, *Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays*, AIDS Res Hum Retroviruses 13:737-42 [1997]).

Localization of HIV-2 DNA and RNA in tissue sections using PCR *in-situ* hybridization (PCR-ISH) and *in-situ* hybridization (ISH): Biopsy and postmortem tissues were preserved in fresh 4% deionized paraformaldehyde, embedded in paraffin wax, and sectioned to 5 μm. The sections were deparaffinized, rehydrated in Tris-buffered saline (TBS; 0.1 M Tris [pH 7.5], 0.1 M NaCl), and digested with proteinase K (20-40 μg/ml, 37°C, for 30-50 min; Sigma). For PCR-ISH detection of HIV-2 *gag* RNA, tissue sections were rehydrated, washed in DEPC water, and treated overnight at 37°C in a RNase-free DNase-1 solution (Boehringer Mannheim), as described previously (Brodie, S.J. *et al.*, *Epizootic hemorrhagic disease. Analysis of tissues by amplification and in situ hybridization reveals widespread orbivirus infection at low copy number*, J Virol 72:3863-3871 [1998a]; Brodie, S.J. *et al.*, *The effects of pharmacological and lentivirus-induced immunosuppression on orbivirus pathogenesis: Assessment of blood monocytes and tissues by in situ hybridization and reverse transcription in situ PCR*, J Virol 72, 5599-5609 [1998b]). The sections were then incubated for 2 min at 70°C, followed by 50 min at 42°C with a mixture of specific SIV antisense primer (SIV3Q2) and MuLV reverse transcriptase (RT-PCR kit; Perkin Elmer, Norwalk, CT). The *gag* cDNA or native *gag* DNA was then reacted with 50 μl of a PCR solution containing 50 pM of the HIV-2 *gag*-specific primers

HIV-2[5Q*i*2], 5'-TTGGATTGGCAGAGAGCCTGTTGGGAT (SEQ ID NO:4); and

HIV-2[3Q*i*2], 5'-TACCCAGGCATTTAAGGTTCGGG (SEQ ID NO:5)

and *taq* polymerase (0.15 U/μl). Primer sequences and thermocycling conditions were as described previously. (Rey-Cuille *et al.* [1994]; Brodie *et al.* [1999b] and [2000b]; Berrey, M.M. *et al.*, *Treatment of primary human immunodeficiency virus type 1 infection with potent antiretroviral therapy reduces frequency of reprogression to AIDS*, J Infect Dis 83:1466-1475 [2001]). The PCR amplification product was detected by ISH using a cocktail of three HIV-2 *gag*-specific oligonucleotides labeled with digoxigenin (DIG) or fluorescein (FAM)-11-dUTP (Boehringer Mannheim, Indianapolis, IN); all were in sense orientation, and internal to the following PCR primer binding sequences

HIV-2[3KD*i*], 5'-AATACCGTCTGCGTCATCTTTTGCC (SEQ ID NO:6);

HIV-2[KD*ii*], 5'-AGCACAGCGACATCTAGCAGCGGACACAG (SEQ ID NO:7); and

HIV-2[KD*iii*], 5'-AGCCGCCTAGCTTATCCAGTGCAGCAA (SEQ ID NO:8).

A 0.8-kb riboprobe was developed for HIV-2/SIV *gag*, as was previously done for HIV-1, SIV, and other animal lentiviruses (Brodie, S.J. *et al.*, *Ovine lentivirus expression and disease: virus replication, but not entry, is restricted to macrophages of specific tissues*, Am J Pathol 146, 1-13 [1995]; Brodie, S.J. *et al.*, *Macrophage function in simian AIDS: Killing defects in vivo are independent of macrophage infection, associated with alterations in Th phenotype, and are reversible with IFN-γ*, J Immunol 153, 5790-5801 [1994]; Brodie, S.J. *et al.* [1998a]; Brodie *et al.* [1999a–b]; Brodie *et al.* [2000b]). The riboprobe was used to localize cells harboring HIV-2 *gag* RNA and to estimate intracellular viral copies.

PCR-ISH and ISH were used to localize latent and low copies of HIV in a variety of tissues and cells (e.g., Figures 3 and 4). When combined with immunohistochemistry, the phenotype of the cell(s) harboring rare viral targets can be identified. Using these combined techniques, HIV-2 DNA and RNA can be localized to specific cell types based on morphology and expression of specific cell surface markers, including $CD21^+$ and $S100^+$ dendritic cells (Brodie *et al.* [1999a]; and Brodie, S.J., *Nonlymphoid reservoirs of HIV-1 replication in children with chronic-progressive disease*, J Leukoc Biol 68, 351-359 [2000e]), macrophages by markers to CD64 and CD68 antigens (Brodie *et al.* [1994], [1995], and [1999a]), and memory and naïve T lymphocytes by detection of $CD45RO^+/CD62L^-$ and $CD45RA^+/CD62L^+$ isoforms (Brodie *et al.* [1999a–b], and Brodie *et al.* [2000a–b]).

Tissue controls for these assays consisted of HIV-2-infected and uninfected CEM-174 cells (e.g., Figure 3) and vaginal and cervical tissues from retrovirus-negative animals. PCR and hybridization controls are the same as described previously (Brodie *et al.* [1998a]; Brodie, S.J. *et al.* [1998b]; Brodie *et al.* [1999a–b], and [2000b]) and included amplification in the absence of *taq* polymerase or specific primers, hybridization with nonsense probes, and incubation with irrelevant isotype-specific antibody. To validate the PCR, test and control samples were prepared and amplified simultaneously with reaction mixtures either containing or lacking *taq* polymerase and specific primers. The presence of HIV-2 RNA and DNA was indicated by a purple cell-associated precipitate (DIG label) or by green fluorescence (FAM label). Images from the representative low-power microscopic fields were transmitted to a computer equipped with a digital imaging board (Brodie *et al.* [1999a]) and the proportion of virus-infected cells determined. Intracellular viral copies were estimated based on total cellular fluorescence.

Isolation and characterization of virus-infected cells from lymph nodes: Activated ($CD45RO^+/CD62L^-$/ $HLA\text{-}DR^+$, plus $CD25^+$, $CD38^+$, $CD69^+$, $CD71^+$, cyclin A^+, and/or $Ki67^+$), quiescent ($CD45RO^+/CD62L^-/HLA\text{-}DR^-$, plus $CD25^-$, $CD38^-$, $CD69^-$, $CD71^-$, cyclin A^-, and $Ki67^-$), and naïve ($CD45RA^+/CD62L^+/HLA\text{-}DR^-/CD25^-/CD38^-/CD69^-/CD71^-$) $CD4^+$ T cells were separated from PBMC and from LNMC using negative selection and magnetic bead removal, combined with fluorescent activated cell sorting (Brodie *et al.* [1994] and [2000b]). Briefly, mononuclear cells were suspended in RPMI 1640, followed by a 1-h incubation (37°C and 5% CO_2) in fibronectin-coated flasks (20 μg/ml) to remove adherent cells. The nonadherent lymphocyte-enriched population were labeled with mAbs to CD8, CD14, CD16, CD19, CD20, and CD21 to enrich for $CD4^+$ T-cells and with mAbs to HLA-DR, CD25 (IL-2R), CD38, CD69, CD71, cyclin A, and Ki67 to remove activated and/or proliferating cells. The cells were incubated with monoclonal antibodies (mAbs) for 30 min at 4°C, washed, and then reacted with secondary mAb conjugated to magnetic microspheres (Dynal, Great Neck, NY) in a bead:cell ratio of 4:1 and incubated at 4°C for 30 min. Rosetted cells were collected by magnetic particle isolation leaving the highly-enriched cell population. The enriched cells were further purified by fluorescent-activated cell sorting using mAbs specific to the lymphocyte subsets CD45RO (memory T cells) or CD45RA/CD62L (naïve T cells). By combining these techniques, >99% of purified cells expressed the specific cell surface markers for the T cell populations defined as activated, quiescent, and naïve. All of the proposed cell surface markers (mAbs) have been used successfully in humans and with varying degree of success in primate cells.

Lymphocyte subset analysis: Fluorescent labeled monoclonal antibodies to lymphocyte surface markers were used to quantitate populations of T cells (CD2+), helper T cells ($CD4^+$), suppressor T cells ($CD8^+$), and B cells ($CD20^+$) in maternal (1 ml) and fetal (150 µl) blood using procedures previously described (Ho RJ, Agy MB, Morton WR, et al. *Development of a chronically catheterized maternal-fetal macaque model to study in utero mother-to-fetus HIV transmission: a preliminary report.* J Med Primatol 1996;25 (3):218-24).

Quantification of HIV-2 RNA in subsets of phenotypically distinct T cells: *In-situ* hybridization was performed in parallel with immunocytochemistry in cell suspension to identify specific $CD4^+$ T-cell subsets that allowed HIV-2 *gag*-pol transcription. Briefly, mAbs specific to cell-surface and intracytoplasmic proteins were applied in combination with HIV-2 RNA-specific oligonucleotide probes to assess HIV-1 transcriptional activity in subsets of $CD4^+$ T lymphocytes (CD45RA or CD45RO) in differing states of activation (CD25, CD38, CD69, CD71, and HLA-DR) and stages of the cell cycle (Ki67 and cyclin A) using a flow cytometry-based detection strategy, as described previously for HIV-1 (Brodie [1999b], [2000a–b]). Mononuclear cells were labeled with fluorochrome (PE, cychrome, PC5, and/or ECD)-conjugated mAbs (PharMingen, San Diego, CA) specific to the cellular antigens described above and then fixed and permeabilized with Permeafix. The cells were then hybridized with a cocktail of fluorescein-labeled oligonucleotide probes spanning open reading frames of HIV-2_{287} *gag-pol* and analyzed by flow cytometry. By evaluating ACH-2 cells containing one integrated copy of HIV-1 DNA and fixed at consecutive time-points following PMA stimulation of viral transcription, the limit of detection of HIV-1 *gag-pol* RNA within a single positive cell was ≤10 copies (Brodie *et al.* [1999b]).

Scanning laser cytometry (LSC): The LSC procedure provides data equivalent to the flow cytometry technique adapted for microscopic slide analysis. The LSC measures four-color fluorescence and light scatter and records the position and time of measurement of each cell. The LSC calculates total cellular fluorescence and can be used to determine a mean signal (viral) copy number within individual cells. It is faster than an image analyzer and shows better detail than a flow cytometer. LSC has been used to differentiate levels of signal intensity in single cells (e.g., Brodie *et al.* [2000a]).

Liquid chromatography-mass spectroscopy (LC-MS) assays to detect drug levels in blood and tissues: A liquid chromatography-mass spectroscopy (LC-MS) assay was used to detect indinavir in plasma

and tissue (homogenate) samples using cyheptamide as internal control prior to extraction with CH_2Cl_2, by methods previouysly described. (e.g., Quian, M. *et al.*, *Metabolism of 3'-azido-3' deoxythymidine (AZT) in human placental trophoblasts and Hofbauer cells*, Biochemical Pharmacology 48, 383-389 [1994a]; Quian, M. *et al.*, *Comparison of intracellular metabolism of AZT in human and primate peripheral blood mononuclear cells*, Antimicrobial Agents and Chemotherapy 38, 2398-2403 [1994b]).

One ml of macaque plasma or 1 mg of lymphoid tissues homogenized in 1ml buffer was extracted with CH_2Cl_2 at pH 8.0 according to Chen et al. (Chen IW, Vastag KJ, Lin JH. *High-performance liquid chromatographic determination of a potent and selective HIV protease inhibitor (L-735,524) in rat, dog and monkey plasma.* J Chromatogr B Biomed Appl 1995; 672 (1):111-7), in the presence of 1 μg cyheptamide as a internal control. After extraction with CH_2Cl_2, the contaminants are removed with a silica column (1 x 4 cm), and the indinavir eluted in 2-propanol: CH_2Cl_2 (1:4 v/v) and dried under N_2. These samples were resuspended in 100 μL, and a 10 μL sample was injected onto an RX-C8 column (2.1 mm x 15 cm column) and eluted isocratically with a mobile phase containing 20 mM ammonium acetate in acetonitrile:water (65:35, v/v) running at 0.38 ml/min. After atmospheric pressure ionization under an electrospray mode, the analytes were detected using selected-ion monitoring (SIM) at m/z 614.7-615.7 *amu* to detect indinavir. Under these conditions, the detection limit was 100 pg, which made it possible to measure drug levels, extract RNA and DNA, isolate cells for detailed analyses of vRNA and vDNA-infected cells in lymph nodes, and fix the tissue for pathological analyses from limited sample size.

Histological analysis of HIV-infected cells: Histologic sections of lymph node were examined using *in situ* hybridization for HIV-2 *gag* RNA. Biopsy and postmortem tissues were preserved in fresh 4% deionized paraformaldehyde, embedded in paraffin wax, and sectioned to 5 μm. The sections were deparaffinized, rehydrated in Tris-buffered saline (TBS; 0.1 M Tris [pH 7.5], 0.1 M NaCl), digested with proteinase K (20-40 μg/ml, 37°C, for 30-50 min; Sigma), and treated overnight at 37°C in a RNase-free DNase-1 solution (Boehringer Mannheim), as described previously. (Brodie SB, Keller MJ, Ewenstein BM, et al. *Variation in incidence of indinavir-associated nephrolithiasis among HIV-positive patients.* Aids 1998;12 (18):2433-7; Diamond C, Brodie SJ, Krieger JN, et al. *Human herpesvirus 8 in the prostate glands of men with Kaposi's sarcoma.* J Virol 1998;72 (7):6223-7).

The sections were then incubated for 5 min at 70°C followed by 4 h at 42°C with antisense RNA probes directed either to SIV (strain mac251; Zhang ZQ, Schuler T, Cavert W, et al. *Reversibility of the pathological changes in the follicular dendritic cell network with treatment of HIV-1 infection.* Proc Natl Acad Sci U S A 1999;96 (9):5169-72) or HIV-2 *gag-pol* (stain 287; *gag* 2639-1080, pol 3473-2306; Galabru J, Rey-Cuille MA, Hovanessian AG. *Nucleotide sequence of the HIV-2 EHO genome, a divergent HIV-2 isolate.* AIDS Res Hum Retroviruses 1995;11 [7]:873-4). The RNA probes were constructed by cloning cDNA into transcription vectors under the control of T7 and SP6 RNA polymerase promoters (pGEM, Promega; Madison, WI). Constructs were linearized and used as templates for *in vitro* transcription to which [^{35}S]-UTP (Amersham Corp., Arlington Heights, IL) was incorporated (~2 x10^6 dpm/μg). After hybridization, the slides were washed in 5X standard saline citrate (SSC), 10 mM dithiothreitol (DTT) at 42°C, 2X SSC, 10 mM DTT, 50% formamide at 60°C, and a 2X RWS buffer (0.1 M tris-HCl (pH 7.5), 0.4 M NaCl, 50 mM EDTA) before digestion with ribonuclease A (25 μg/ml) and T_1 (25 units/ml) in 1X RWS. After washing in RWS, 2X SSC, and 0.1X SSC, sections were dehydrated in graded ethanol containing 0.3 M ammonium acetate and then air-dried and dipped in Kodak NTB-2 emulsion, exposed at 4°C, developed, and lightly counterstained. HIV-2 RNA was detected after autoradiographic exposures of 24 and 96 h.

The presence of viral RNA was indicated by deposition of silver grains on top of cells or as aggregates within lymph node germinal centers (Figure 7) at a frequency statistically greater than background, as determined using Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD). Nonspecific background was determined in two ways with equivalent results. Silver grains were counted over 100 germinal center cells in tissue sections from mock-inoculated uninfected animals after hybridization to the HIV-2 or SIV antisense probe, or over cells from the tissues of infected monkeys after hybridization to noncomplimentary 'sense' RNA probes. In both cases, the average background was 0.3 grains per cell for the 24 h exposure. Additional controls consisted of tissue sections, with and without protease treatment, and antisense RNA from the visna virus *gag* gene. (Brodie SJ, de la Rosa C, Howe JG, et al. *Pediatric AIDS-associated lymphocytic interstitial pneumonia and pulmonary arterio-occlusive disease: role of VCAM-1/VLA-4 adhesion pathway and human herpesviruses.* Am J Pathol 1999;154 (5):1453-64; Brodie SJ, Pearson LD, Zink MC, et al. *Ovine lentivirus expression and disease. Virus replication, but not entry, is restricted to macrophages of specific tissues.* Am J Pathol 1995;146 (1):250-63).

The Poisson probability that x number of grains differs from a background average of m is $P = (m^x \times e^{-m})/x$. For a cell with ≥5 grains over background, the probability that the cell is infected is >0.99. Using previously validated back-calculation methods (Haase AT, Henry K, Zupancic M, et al. *Quantitative image analysis of HIV-1 infection in lymphoid tissue.* Science 1996;274 (5289):985-9; Haase AT, Stowring L, Harris JD, et al. *Visna DNA synthesis and the tempo of infection in vitro.* Virology 1982;119 (2):399-410; Zhang ZQ, Schuler T, Cavert W, et al. *Reversibility of the pathological changes in the follicular dendritic cell network with treatment of HIV-1 infection.* Proc Natl Acad Sci U S A 1999;96 (9):5169-72). In the study described herein, one silver grain approximated 2 copies of HIV-2 RNA. The amount of viral RNA within germinal centers was also calcualted. Silver grain counts were averaged per six consecutive LN germinal centers (50X microscopic fields) at the 24 h exposure. Similar results were achieved with both SIV and HIV-2 RNA probes.

Example 2

The Effect of pH on the Ability of Drugs to Associate to Lipid Bilayer

Figure 1A illustrates the pH-dependent incorporation of indinavir within lipid-associated complexes. With lipids containing phosphatidyl choline (egg): cholesterol (3:1 molar ratio) and lipid to indinavir (5:1 molar ratio), small unilamellar liposomes were prepared with phosphate-buffered saline at indicated pH value. They were sonicated to achieve 55 ± 5 nm in diameter. Subsequently, the % lipid-association was determined by separating free from lipid-associated drug by size-exclusion column chromatography. Data expressed were means of duplicate preparations for indicated pH value.

It has been reported that changing the pH of indinavir from pH 3 to pH 7 results in a 1000-fold decrease in its aqueous solubility as provided in Table 1A (Lin *et al., pH-dependent oral absorption of L-735,524, a potent HIV protease inhibitor, in rats and dogs*, Drug Metab Dispos 23:730-735 [1995]). The effect of pH on the ability of indinavir to incorporate or associate with lipid (i.e., liposome) membranes in forming lipid-drug complexes was determined. At a lipid-to-drug ratio of 5:1 (m/m), practically all (85–95%) of the drug in the preparation was found to be associated with liposome at pH 7.4, as illustrated in Figure 1A. At lower pH values (e.g., pH 4), where aqueous solubility of the drug was higher, a much lower proportion (< 30%) of the drug was incorporated into the lipid bilayer of liposomes. Since physiological pH is 7.4, and because biological fluids are highly buffered, lipid-associated drugs are expected to remain stable under these conditions. The lipid-indinavir complexes formed and maintained at pH 7.4 were used for the

subsequent pharmacokinetic studies. Under this set of conditions, lipid-associated indinavir exhibited a diameter of 69 ± 7 nm as provided in Table 1B.

Figure 1B illustrates the pH-dependent release of indinavir from lipid-associated complexes *in vivo*. The pH dependence on the lipid and drug association is also consistent with the observation that the lipid-associated drug can be released in a pH-dependent manner. Because of the stringent active-site-binding requirement and pharmokinetic profiles, most of the clinically used anti-HIV protease inhibitors exhibit a profile with similar lipophilicity, which is also pH-dependent. We found that saquinavir and nelfinavir exhibit a high degree of lipid association, similar to indinavir as provided in Table 1B.

Table 1A. pH-dependent Effects of Indinavir Solubility and Lipophilicity

pH	Solubility in aqueous	LogP (lipophilicity)
3.5	50 mg/ml	0
5	0.1 mg/ml	1.8
7	0.01 mg/ml	3

From *Lin et al., 1995 Drug Metabolism and Disposition 23:730-5*

Table 1B. The Characterization of Anti-HIV drugs Associated with Lipid Membranes

Anti-HIV Drug	Class of Drug	Degree of Lipid Association (% total)	Size in Diameter (nm)
Indinavir	Protease inhibitor	97.5 ± 2.5	69 ± 7
Saquinavir	Protease inhibitor	98.8 ± 7.5	159 ± 36
Nelfinavir	Protease inhibitor	100.5 ± 9.8	123 ± 16
Viread®	RT inhibitor[a]	4.2 ± 0.7	60 ± 10

[a]Reverse transcriptase inhibitor, adenosine nucleotide analog.

Example 3

Comparison of Indinavir Concentrations in Human Lymph Node

and Peripheral Blood Mononuclear Cells (PBMCs)

Figure 2A illustrates a typical time course for a virus load, and the $CD4^+$ T cell profile of macaques infected with HIV-2_{287}. A representative macaque (pregnant macaque at 140 d gestation) was inoculated with 10 TC_{ID50} of HIV-2 (IV) and virus-infected PBMC (shaded points), and $CD4^+$-T cells (unshaded points) were monitored every other day until delivery of the infant by C-section. To determine the indinavir concentrations in mononuclear cells of lymph nodes (LNMC) in relation to that of blood (periphery), we employed a highly sensitive liquid chromatography-mass spectroscopy (LC-MS) assay to estimate indinavir content in 5 x 10^5 of each type of cell. The matched peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells (LNMC) data (collected from blood and lymph node respectively) from 3 HIV^+ patients treated by HAART, including indinavir, indicated that the indinavir concentrations in LNMC were much lower than in PBMC (Table 2). These data indicated that indinavir concentration in lymph node mononuclear cells was much lower than in their blood counterpart, with the ratio ranging from 0.23 to 0.35 (i.e., less than *unity or 1*). Assuming that each mononuclear cell has a volume of $4x10^{-9}$ ml^3 (a value estimated for mammalian cells in Alberts *et al.*, *Macromolecules; structure, shape, and information*. In "Molecular Biology of the Cell", 3rd edition, Garland Publishing, Inc., NY., pp 89-90 [1994]), the intracellular indinavir concentration for Patient JS1166's PBMC was calculated as 0.86 μg/ml, a value similar to the plasma concentration (0.616 μg/ml). The similar indinavir concentration observed in plasma and PBMC is consistent with the data of Lin *et al.* (Lin *et al.*, *Species differences in the pharmacokinetics and metabolism of indinavir, a potent human immunodeficiency virus protease inhibitor*, Drug Metab Dispos 24:1111-20 [1996]), demonstrating that indinavir in plasma can equilibrate readily with erythrocytes in blood. More importantly, these data indicate that indinavir concentrations in lymph nodes, particularly mononuclear cells, are much lower than in plasma and blood cells. Therefore, enhanced drug delivery to lymphoid organs, particularly to lymph nodes, should significantly improve control of viral replication in lymphoid tissues.

Table 2. Indinavir Concentration in Mononuclear Cells of Peripheral Blood, Lymph Nodes, and Plasma*

Patient ID	Plasma (μg/ml)	PBMC (ng / $5x10^5$ cells)	LNMC (ng / $5x10^5$ cells)	LNMC ratio PBMC
JS1166	0.616	1.72	0.60	0.35
JA1216	NA	1.88	0.53	0.28
SS1196	NA	0.53	0.124	0.23

*Lymph node mononuclear cells were isolated from lymph node biopsy collected at the same time when the blood samples were collected to isolate plasma and PBMC from the same patients. Cellular indinavir concentrations were determined by LC-MS using indinavir extracted from 50,000 cells. Data expressed were mean of duplicate extractions of each sample.
NA: no sample available.

Example 4

Characterization of a Highly Pathogenic Strain of HIV-2 Infection in Macaques

In more than 20 HIV-2_{287}-infected pregnant macaques (*Macaca nemestrina*), a prominent viremia phase was observed that peaked within 2 weeks, detected as corresponding peaks in virus-infected cells in blood and free virus (quantitated by QC-RNA-PCR) in plasma, and a subsequent rapid $CD4^+$-T cell decline within 3 weeks post-infection (Ho *et al.*, *Development of a chronically catheterized maternal-fetal macaque model to study in utero mother-to-fetus HIV transmission - a preliminary report. An invited article*, J Medical Primatol 25:218-24 [1996]). A typical time course of disease progression following HIV-2_{287} is graphically presented in Figure 2A in terms of virus load and $CD4^+$ T-cell depletion. In Figure 2A, a representative macaque (pregnant macaque at 140 d gestation) was inoculated with 10 TC_{ID50} of HIV-2 (IV) and virus-infected PBMC (shaded points), and $CD4^+$-T cells (unshaded points) were monitored every other day until delivery of the infant by C-section.

Figure 2B illustrates the analysis of plasma for a viral RNA profile of 27 macaques that were infected with 50 TC_{ID50} HIV-2_{287}. Each data point represent a sample collected from each animal. Figure 2B indicates that virus was detectable in the plasma at day 4 and reached a peak value of approximately 5×10^7 copies/ml between days 10 and 14 after infection. The viral load after the acute phase of infection ("viral set-point") was reached at day 21 and remained detectable consistently at approximately 10^6 copies/ml thereafter. These results also demonstrate the rapid progression and highly reproducible nature of HIV-2_{287} infection in pig-tailed macaques. These properties are highly desirable for antiviral drug studies, because a much shorter time frame is needed with perhaps greater statistical power to detect therapeutic effects.

To determine the time course of virus levels in lymph nodes, 27 macaques were infected intravenously with HIV-2_{287} at a dose of 50 TC_{ID50}. Three animals were sacrificed on each of the following days after infection: 0.5, 1, 2, 4, 6, 10, 14, 21, 28-30. The following tissues were collected: peripheral blood mononuclear cells, bone marrow, spleen, ileocecal lymph nodes, inguinal lymph nodes, axillary lymph nodes, mesenteric lymph nodes, deep pelvic lymph nodes, submandibular lymph nodes, and thymus. Viral load in each of these tissues was determined by

quantitative co-culture with PHA-activated $CD8^+$ cell-depleted human PBMC. The results indicated that productively infected cells were first detectable in all tissues between 4–6 days after infection at a level of 1-100 infectious cells per million. The viral load peaked between days 10–14 reaching a level of approximately 10^3-10^4 infected cells per million (i.e., 0.1–1% of cells were productively infected). This level decreased after acute infection, but remained detectable at 10–1000 infected cells per million between 21–30 days after infection.

Example 5

The Effects of Lipid Association on the Ability of Indinavir to Inhibit HIV-2_{287} Replication

Figure 3 illustrates the concentration-dependent inhibition of HIV-$_{287}$ replication by free (not lipid-associated) and lipid-associated indinavir. HIV-2_{287}-infected CEM-174 cells (0.01 multiplicity of infection [MOI]) were incubated with the indicated concentrations of indinavir, either in free (open symbols) or lipid-associated (closed symbols) formulation and drug effects on virus replication are expressed as mean % infected cells of quadruplicate samples that were assayed for the presence of p27 core antigen of HIV-2. Under these conditions, all the control samples without drugs were positive for viral replication. The effective concentrations that produce half the maximum anti-HIV activity (EC_{50}) were determined based on non-linear regression of each set of data, representing the frequency of replication.

To determine anti-HIV activity of lipid-associated indinavir, 5 x 10^5 CEM-174 cells were infected with 5 x 10^3 TC_{ID50} (multiplicity of infection, MOI = 0.01) HIV-2_{287} in 2 ml RPMI 1640 tissue culture medium containing 1% fetal calf serum for 1 hr at 37°C. After unabsorbed virus was removed by washing the cells with medium, 100 μl of suspensions containing 10^4 infected cells were transferred to flat-bottom, 96-well microtiter plates containing 100 μl of serially diluted (0-15 μM) indinavir, either in free or liposome-associated formulations. After incubating the cells at 37°C in RPMI 1640 containing 10% fetal calf serum for 4 days (optimum detection time), the presence of virus-infected cells was determined visually by the presence of syncytia and was subsequently confirmed by ELISA detection of the presence of HIV-2 antigen. Experiments were repeated on at least two different days with each determination done in quadruplicate samples, and the data presented in Figure 3 are the mean % virus-infected cells. Regression analysis estimated the EC_{50} (50% effective inhibitory concentration) value for lipid-associated indinavir to be 0.01-0.025 μM, and 0.05-0.08 μM for free indinavir. These data imply that lipid-associated indinavir is about 3- to

6-fold more potent than free drug in inhibiting HIV-2_{287} replication. A similar degree of enhancement was recorded for HIV-1_{LAI}-infected human PBMC. Figure 6 shows the concentration-dependent inhibition of HIV-1_{LAI} replication by the free and lipid-associated indinavir.

Example 6

A Plasma Time Course Profile of Free versus Lipid-Associated Indinavir in Macaques

Figure 4 illustrates a time course for plasma concentration of indinavir following the subcutaneous administration of lipid-associated and non-lipid-associated indinavir within macaques. Young adult male macaques were given either free or liposome-formulated indinavir at 10 mg/kg body mass per dose, and plasma drug concentrations were determined by HPLC assay. Data expressed were means ± SD for animals injected with free (open squares, n = 4) and liposome-formulated indinavir (other symbols; n = 4). Young adult (5–6 kg body mass) macaques that were administered subcutaneously (SC) with either free or lipid-associated indinavir (SC) at 10 mg/kg body mass per single dose. Free indinivir, solubilized in DMSO and phosphate buffer suspension, produced a plasma drug concentration peak at about 0.5–1 hr, and rapidly cleared the drug to below the limit of detection in plasma by 6 hr (Figure 4). In contrast, lipid-associated indinavir produced a peak plasma concentration about 10-fold lower than free drug, and sustained this plasma level beyond 10 hr. When a second dose was given after a 30-day washout period, a significant amount of drug (> 20 ng/ml) remained in plasma beyond 24 hr (Figure 4; profile of *liposome-1 and -2*). Animals labeled as liposome-3 (M98165) and -4 (J98328) were previously infected with HIV-2287 and, hence, allowed collection of the visceral lymph nodes for drug analysis. The data are presented in Table 3 provided in Example 7 below.

Example 7

The Effect of Lipid-Drug Complexes

on Enhanced Accumulation of Indinavir in Lymph Nodes

In some experiments, lipid-associated indinavir (10 mg/kg body mass) was administered to two additional HIV-2_{287}-infected macaques, and inguinal lymph nodes were harvested at 6, 24 and 16 or 28 hrs. Drug concentration was measured in blood as well as lymph nodes. Time-course plasma drug concentrations of these two animals are presented in Figure 4

(*liposome-3 and -4*).

In contrast, in two macaques that were administered lipid-associated indinavir, the lymph-node-to-plasma ratio ranged from 2.5- to 22.7-fold between 6 and 28 hrs post-administration provided in Table 3. The variability in drug accumulation between lymph nodes may be due to the limited flow and diffusion rates of the lipid-drug particles within the lymphatic systems. The variability can be reduced by administration of the lipid-indinavir in multiple sites or repeated dosing schedule. Even at 24 to 28 hrs, 20–30 ng/ml of indinavir was available in blood. Given the *in vitro* ED_{50} of indinavir, 0.001-0.025 μM or 7-17 ng/ml against HIV-2_{287} for lipid associated form, and 42-56 ng/ml (0.06-0.08 μM) for free drug, these values are within its acceptable, but low, therapeutic range. Hence, the dose of indinavir should be increased 2- to 4-fold (20–40 mg/kg body mass) to achieve higher plasma drug levels to produce maximum effect on virus load reduction.

Three HIV-2_{287}-infected macaques, administered 25 mg/kg body mass oral indinavir, showed minimal levels of drug presence in either axillary or inguinal lymph nodes provided in Table 4. In contrast, in animals administered with lipid-associated indinavir, we found that the lymph node-to-plasma ratio ranged from 2.5- to 22.7-fold between 6 and 28 hrs post-administration in two animals (Table 3). Data collected from 25 mg/kg body mass oral indinavir to HIV-infected macaques exhibited no detectable indinavir in plasma or lymph nodes beyond 8 hr; more importantly, at any given time point, lymph node-to-plasma drug ratios *never exceeded unity* (Table 4). Oral indinavir administration to rats by Lin *et al.* indicates that while [^{14}C]-labeled indinavir rapidly distributed into the mesenteric lymph, it was cleared from the lymphatic system at a much faster rate than from plasma. (Lin *et al., Species differences in the pharmacokinetics and metabolism of indinavir, a potent human immunodeficiency virus protease inhibitor*, Drug Metab Dispos. 24:1111-20 [1996]). These data imply that lipid-associated indinavir provides enhanced lymph node accumulation of drug at levels that cannot be achieved by free drug administration; additionally, lipid association can produce sustained therapeutic levels in blood for a much longer duration.

Furthermore, these data also imply that lipid-drug complexes are sufficiently stable *in vivo*. If lipid-drug complexes were dissociated (to release free drug) at injection sites, free drug diffused or perfused to lymph nodes would produce much lower concentrations than those found in blood, and would never reach higher concentrations than in blood. In this case, lymph node to blood ratios would be around or less than one.

Table 3. Indinavir Accumulation in Selected Lymph Nodes of HIV-infected Juvenile Macaques after Subcutaneous Administration of 10 mg/kg Body Mass Lipid-associated Indinavir

Animal ID	Lymph node	Time (hr)	Lymph node (indinavir) (ng/ml)	Plasma (indinavir) (ng/ml)	Lymph node to plasma ratio
M98165	Inguinal	6	1004.3	44.2	22.7
	Inguinal	28	109.2	31.8	3.4
	Mesenteric	28	158.8	31.8	5.0
	Ileocecal	28	1035.2	31.8	32.5
	Axillary	28	78.3	31.8	2.5
J98328	Inguinal	24	147.6	20.7	7.1
	Inguinal	26	130.4	20.8	6.3
	Mesenteric	26	338.3	20.8	16.3
	Ileocecal	26	145.8	20.8	7.0
	Axillary	26	51.6	20.8	2.5

Table 4. Indinavir Accumulation in Select Lymph Nodes of HIV-infected Macaques at Indicated Time Point after Oral Administration of Free Indinavir (25 mg/kg body mass) in Solution

Animal ID	Lymph node	Time (hr)	Lymph node (indinavir) * (ng/ml)	Plasma (indinavir) (ng/ml)	Lymph node to plasma ratio
94079	Inguinal	3.25	0.283	9.8	0.03
	Axillary	3.25	0.128	9.8	0.01
94094	Inguinal	2	0.056	5.2	0.01
	Axillary	2	0.084	5.2	0.02
94096	Inguinal	3.75	0.481	39.8	0.01
	Axillary	3.75	0.141	39.8	3.5×10^{-3}

Example 8

The Effect of Lipid-indinavir Complex on HIV-2_{287} Infected Macaques

Figure 5 illustrates the changes in plasma virus load and the $CD4^+$ T-cell profile monitored in two HIV-2_{287}-infected macaque at 25 weeks post-infection. Figure 5A (macaque ID M98311) and Figure 5B macaque (K98158) show the time-course of plasma viral RNA level (closed symbols) and $CD4^+$ T-cell count (open symbols). Each macaque was injected subcutaneously with a single daily dose of 20 mg/kg body mass of lipid-indinavir on 10 days over a 14-day period. Two macaques infected with HIV-2_{287} (about 30 weeks post-infection), exhibiting different degrees of disease progression, were treated with lipid-indinavir complexes over a 14-day period. At initiation of indinavir therapy, the $CD4^+$ T-cell concentrations in one animal had declined below 100 (or 3% of total lymphocytes), and this animal did not reverse $CD4^+$ T-cell decline in response to the drug therapy. This observation is consistent with that of human subjects under HAART where patients with $CD4^+$ T-cells below 200 are less likely to respond to drug therapy. The second animal exhibiting $CD4^+$ T-cells above 200 levels (at initiation of therapy) responded to the lipid-indinavir therapy. Analysis of virologic and T-cell responses indicated that even with the dose that was not optimized (10 single daily doses of 20 mg/kg body mass/day given over a 14-day period), lipid-indinavir significantly reduced the plasma virus load by day 6. The reduction in plasma virus load was reflected in $CD4^+$ T-cell profile that rebounded by day 5, and sustained at this new level (> 25%) even after cessation of the drug therapy at day 13.

About 20-fold higher indinavir concentration (1.2 μg/g in lymph nodes vs. 50 ng/ml in plasma at 14 hr) was achieved in axillary lymph nodes, distal to the lower scapular subcutaneous injection site at 13.3 hrs post-injection in the second animal. These data imply that subcutaneously administered lipid-indinavir complexes may distribute and accumulate in lymphoid tissues throughout systemic circulation and provide sustained lymph node as well as plasma indinavir levels. Also, the results confirm the data presented in Table 3. Sustained plasma drug levels were evident as shown by the continued presence of plasma indinavir (26 ng/ml) on day 17, more than 3 days (or 85 hrs) after the last dose of lipid-indinavir, was given to the second animal.

With four additional HIV-2_{287}-infected animals at various stages of disease progression, we compared the effects of lipid association on the ability of indinavir to alter the pathogenesis. Plasma cholesterol level was monitored to evaluate the effects of cholesterol given as a part of lipid formulation. As shown in Table 5, while these macaques were infected with varying

doses of HIV-2_{287} and exhibited different degrees of peak plasma virus load, animals treated with lipid-associated indinavir for two weeks showed an increase in $CD4^+$T cells, while the same pattern was not observed with animals treated with free indinavir. Furthermore, the additional dose of cholesterol given in the lipid-drug formulation did not alter the overall plasma cholesterol level.

Table 5. Effects of Indinavir Therapy on $CD4^+$T cell and Cholesterol Levels in HIV-2-Infected Macaques[a]

Macaques	Initial HIV Inoculation Dose[b]	Peak viral RNA in Plasma (copies/mL)[c]	$CD4^+$ T cell (per μL)		Plasma Cholesterol (mg/dL)	
			Before[d]	After[d]	Before[d]	After[d]
Lipid-free indinavir treated						
215	1	3.2×10^6	1946	1473	121	125
283	0.1	1.1×10^5	1650	1051	193	188
Lipid-associated indinavir treated						
052	1000	2.3×10^7	242	527	175	169
225	0.1	5.3×10^5	875	1707	124	136

[a]Macaques, previously inoculated with HIV-2 287 at 33 weeks post infection, were treated with 22 mg/kg daily doses of indinavir for 14 doses. Animals 215 and 283 were subcutaneously treated with soluble indinavir formulation while 052 and 225 were treated with lipid-associated indinavir formulation. These animals had not been treated previously with any anti-HIV therapy.

[b]Macaques were inoculated at indicated dose of HIV-2_{287}. All animals except 052 were inoculated by intravenous route. Macaque 052 was inoculated by intravaginal route as a part of a viral dose titration study. About a thousand-fold higher dose of virus is required, typically, to produce HIV infection in these animals.

[c]Peak plasma viremia was observed within 2-3 weeks post viral inoculation and analyzed with an RT- QPCR and expressed as copies/mL.

[d]The $CD4^+$ T-cell concentration and plasma cholesterol levels were measured before and after indinavir drug therapy.

Example 9

Lipid Association Enhances the Ability of Indinavir to Inhibit HIV-1 Replication in Human Peripheral Blood Mononuclear Cells

Figure 6 shows the concentration-dependent inhibition of HIV-1_{LAV} replication by the free and lipid-associated indinavir. HIV-1-infected PBMCs were incubated with indicated concentrations of indinavir either in free (circles) or lipid-associated (squares) formulation, and drug effects on virus replication are expressed as mean % inhibition of duplicate samples that are assayed for the presence of HIV-1 p24 antigen. Under these conditions, all the control samples without drugs were positive for viral replication. The effective concentrations that produce half the maximum anti-HIV activity (EC_{50}) were determined based on non-linear regression of each set of data, representing the frequency of replication.

To evaluate the role of lipid formulation on indinavir's potency against HIV-1 replication, $CD8^+$ cells depleted, human peripheral blood mononuclear cells (PBMCs) (previously stimulated with PHA and IL-2, as described in Example 1, were infected with HIV-1_{LAV}. The10^4 HIV-1 infected PBMCs were exposed to 200 μl of serially diluted (0-15 μM) indinavir suspensions in free or liposome-associated formulations expressing either net positive or negative charge. Virus replication was assessed by measuring HIV-1 p24 antigen presence in the culture supernatant. Experiments were repeated on two different occasions with each determination done in duplicate, and the data presented are the mean % inhibition (Figure 6). Regression analysis estimated the EC_{50} value for lipid-associated indinavir to be 0.02-0.03 μM, and >0.15 μM for free indinavir. Even at 15 μM, free indinavir did not exhibit 100% inhibition. These data implies that lipid-associated indinavir is more potent than free drug in inhibition of HIV-1 replication.

Example 10

Liposome-indinavir Complexes Accumulate in Lymphoid Tissues and Reduce HIV Viral Load in Infected Macaques

In additional experiments, data were collected from four HIV-2_{287}-infected macaques (*Macaca nemestrina* at 30 weeks post-infection), treated with 20 mg/kg/day subcutaneous lipid-indinavir complexes or free indinavir for 14 days. Similar to data presented in Table 3, about 20-fold higher indinavir concentration was achieved in axillary lymph nodes, distal to the lower scapular subcutaneous injection sites in lipid-indinavir-treated animals (at 13.3 hrs post-injection, data not shown). These results imply that lipid-indinavir complexes accumulate throughout lymphoid tissues. Sustained plasma drug levels were detectable even a few days after lipid-indinavir administration. At 3 days after cessation of lipid-inidnavir in the two animals, about 30 ng/ml plasma indinavir was detected.

Drug levels in the two animals treated with free indinavir subsided below detectable levels by 4-5 hrs.

Virologic and T-cell analyses indicate that even with this unoptimized lipid-indinavir dose (14 X [20 mg/kg body mass/day] dose given over 14 days) indinavir treatment had significantly reduced the plasma virus load by day 6. Animals treated with free indinavir did not exhibit a significant virus load reduction under these conditions. The reduction in plasma virus load of animals treated with lipid-indinavir was reflected in $CD4^+$ T-cell profile that rebounded by day 5, and sustained at this new level (> 25%) even after cessation of drug therapy (day 20). These data are similar to those presented in Figure 5.

Figure 7 shows *in situ* hybridization analysis of lymph node sections in indinavir treated animals with a [^{35}S]-labeled HIV-2_{287}-specific probe. HIV-2-infected macaques were treated with formulations of 20 mg/kg body mass of lipid-indinavir complexes [lipid-complexed (IND)] (Figure 7A, 052) and free indinavir [lipid-free IND] (Figure 7B, 215) for 14 days, and lymph nodes were collected by necropsy on day 20. Only animals treated with lipid-free drug showed evidence of HIV-2 RNA in lymph node germinal centers (arrows). Analysis was performed on viral RNA expressing cells (by *in-situ* hybridization to detect viral RNA) in axillary and mesenteric lymph nodes of these four HIV-infected animals; two treated with lipid-indinavir complexes (animals 052 and 225) and two with lipid-free IND (macaques 215 and 283). Examination of axillary and mesenteric lymph nodes from the four HIV-2_{287}-infected animals, two treated with lipid-associated indinavir (animals 052 and 225) and two with lipid-free indinavir (animals 215 and 283) was performed. Representative photomicrographs of axillary lymph node sections hybridized with an [^{35}S]-labeled-HIV-2_{287} RNA probe are shown in Figure 7. Only the animals treated with free drug showed aggregates of HIV-RNA in lymph node germinal centers, the sites to which follicular dendritic cells are restricted. Both axillary and mesenteric lymph nodes were positive for HIV-2 RNA in these two animals provided in Figure 7B. Macaque 215 had slightly higher concentrations of viral RNA in its lymph nodes (13,290 ± 1,450 *gag-pol* RNA copies/50X field; Figure 7B), compared to macaque 283 (8,134 ± 890 *gag-pol* RNA copies/50X field). Free indinavir-treated animals showed only slightly less accumulation of viral RNA than the untreated HIV-infected control animals (9,222 ± 1,100 *gag-pol* RNA copies/50X field; $P >$ 0.05). In contrast, lymph nodes from animals treated with lipid-indinavir showed much reduced viral RNA (Figure 7A), with silver grain counts ranging from 337 to 1,280 (mean, 765 ± 94 grains) per 50X microscopic field ($P < 0.001$). In contrast, lymph node samples

from animals treated with lipid-indinavir showed much reduced viral RNA by *in situ* hybridization provided in Figure 7A.

Collectively, these data indicate that lipid-indinavir complexes are highly efficient in reducing the plasma virus load *in vivo*, and in reversing the $CD4^+$ T-cell decline (due to natural course of HIV-2_{287} infection). The indinavir delivered in lipid-indinavir complexes provided sustained and high drug concentrations in lymph nodes. The *in-situ* virus analysis of lymph nodes clearly indicates that treatment with lipid-indinavir complex, but not free indinavir treatment, had significantly reduced virus load in lymph nodes. Also, lipid-indinavir did not appear to influence lymph node structure, and therefore, this strategy may greatly reduce dose-limiting toxicity observed with systemic (plasma) exposure of high-dose indinavir. These data add significantly to the likelihood of success in achieving our goal to determine whether subcutaneous (SC) lipid-indinavir treatment is effective in reducing virus load in plasma as well as lymphoid tissues in HIV-infection and pathogenesis.

The foregoing description, for purposes of explanation, used specific nomenclature to provide a thorough understanding of the invention. However, it will be apparent to one skilled in the art that the specific details are not required in order to practice the invention. The foregoing descriptions of specific embodiments of the present invention are presented for purpose of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed. Obviously many modifications and variations are possible in view of the above teachings. The embodiments are shown and described in order to best explain the principles of the invention and its practical applications, to thereby enable others skilled in the art to best utilize the invention and various embodiments with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the following claims and their equivalents: